Patent Application of

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For

TITLE OF THE INVENTION

De novo synthesized plasmid, methods of making and use thereof

CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable

REFERENCE TO SEQUENCE LISTING

A sequence listing of this invention is submitted on paper after the Declaration. The sequence listing was generated by software Patent-In 3.1.

BACKGOUND OF THE INVENTION

This invention relates to recombinant DNA technology for making and using a novel plasmid. More particularly it relates to methods for making and using a de novo synthesized plasmid with defined copy number and selection marker. This de novo synthesized plasmid is useful in making other plasmids and cell strains containing these plasmids.

Plasmids are extrachromosomal circular double stranded DNA loops that are transferable from one bacterium to another. Plasmids replicate independently of that of the chromosomal DNA of a host cell. The number of plasmid in a host cell is called the copy number of the plasmid. A given plasmid may be present in a low copy number or a high copy number inside a bacterial cell. The copy number is a genetic characteristic of the replication origin of each plasmid. The replication origin of a plasmid determines its copy number.

The type of replication origin divides plasmids into different incompatibility groups. Different plasmids of same incompatibility group cannot stably coexist in one bacterial cell. On the other hand, plasmids of different incompatibility groups can stably coexist in one bacterial cell. For example, different plasmids of the ColE1-type replication origin (such as plasmids of the family's pBR, pUC and the like) cannot coexist stably in one bacterial cell. Different plasmids of p15A-type replication origin (such as plasmids of the family pACYC and the like) cannot coexist stably in one bacterial cell. However a plasmid of the ColE1-type replication origin and a plasmid of p15A-type origin can coexist stably in one bacterial cell because they belong to different incompatibility groups.

In addition to replication origin, plasmids often contain selection marker genes. A selection marker gene normally allows for phenotypic selection in transformed host cells. Some selection marker genes encode proteins conferring host cell resistance to certain antibiotics. Examples of antibiotics resisted by proteins encoded by selection marker genes include ampicillin, tetracycline, chloramphenicol, kanamycin, gentamycin, rifampicin, spectinomycin, streptomycin, and the like.

Plasmids are useful in DNA cloning, DNA amplification, gene expression, gene therapy, DNA immunization, and like biomedical applications. Substantial efforts have been made to construct numerous plasmids. All of the plasmids made from prior arts are modifications of the plasmids previously obtained from natural sources or recombinant sources. These plasmids inevitably contain some DNA sequences with unknown and/or

undesirable function (junk sequences). These junk sequences consume cellular resources and contribute to cellular energy drains. In some cases, the junk sequences have detrimental effects on the plasmid applications. Therefore a novel plasmid, which contains relevant sequences, is generated from de novo synthesis and minimizes the junk sequences, will be valuable in the biomedical applications.

SUMMARY OF THE INVENTION

In general, the present invention provides a novel plasmid. More specifically, the invention provides a de novo synthesized plasmid comprising at least a replication origin and a selection marker gene wherein

(a) the replication origin contains sequences relevant to autonomous plasmid replication in a host cell; and

(b) the selection marker gene contains sequences relevant to the selection of the plasmid in a host cell.

Wherein the de novo synthesized plasmid is not modified from the plasmid previously obtained from natural or recombinant sources. Wherein the replication origin allows the autonomous plasmid replication in a host cell. Wherein the selection marker gene encodes a product indicative of plasmid maintenance in a host cell.

The present invention further provides a method of preparing the de novo synthesized plasmid combined from at least two DNA fragments comprising:

(a) preparing a linear replication origin DNA fragment;

(b) preparing a linear selection marker gene DNA fragment;

(c) combining the DNA fragments prepared from steps (a) and (b) to form a circular de novo synthesized plasmid;

(d) introducing the plasmid made from step (c) into a host cell; and

(e) selecting the plasmid with appropriate replication origin and selection marker from transformed host cells.

Wherein any DNA fragment alone used for combining the de novo synthesized plasmid cannot confer both autonomous DNA replication and selection to a plasmid. In one preferred embodiment, the linear DNA fragment is prepared from polymerase chain reaction (PCR). In another preferred embodiment, the linear DNA fragment is prepared from restriction digestion.

The present invention also provides a method of using the de novo synthesized plasmid comprising:

(a) linearizing the de novo synthesized plasmid;

(b) inserting one or more functional DNA fragments into the plasmid to make other plasmids;

(c) introducing the plasmids made from step (b) into host cells;

(d) selecting the plasmids and the host cells with desired properties; and

(e) using the plasmids and the host cells for biomedical applications

Wherein the de novo synthesized plasmid is linearized by restriction digestion. Wherein the de novo synthesized plasmid is linearized by PCR. Wherein the functional DNA fragments encode a promoter, a regulatory sequence, a ribosome binding site, restriction

sites, a terminator, a polypeptide, a replication origin, a selection marker gene, and the like useful DNA sequences. Wherein the desired properties are plasmid replication, selection, and other properties added by functional DNA fragments inserted from step (b). Wherein the biomedical applications are DNA cloning, DNA amplification, gene expression, gene therapy, DNA immunization, and the like.

Further objects and advantages of the invention will become apparent from a consideration of the drawings and ensuing description.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Schematic diagrams of construction of a de novo synthesized plasmid p4T. The arrow indicates the direction of transcription.

FIG. 2. Plasmid p4T is a high copy number plasmid. The replication origin of p4T is derived from low copy number replication origin p15A. However its copy number is comparable to high copy number plasmid pUC19 derived plasmids p3A, p3T, and p4K. The name of each plasmid is indicated above each lane of the gel. The arrow on the gel indicates approximate position of the linearized plasmid.

FIG. 3. Schematic diagram of construction of plasmids or p4TI, p4TI3, and p4TI3E from a de novo synthesized plasmid p4T. The arrow indicates the direction of transcription.

FIG. 4. Protein expression studies. Experimental results show expression of proteins by plasmids generated from de novo synthesized plasmids p4TI3E and p2CXL. Fig. 4A shows both proteins EL and ES were expressed from induced sample containing p4TI3E. Figure 4B shows protein XL was expressed from induced sample containing p2CXL.

FIG. 5. Host cells containing p4CXL aid the solubility of AL. Protein AL is not soluble when it is expressed by itself. It becomes soluble when it is expressed in the host cell containing p4CXL, which is generated from a de novo synthesized plasmid p4C.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to a novel recombinant plasmid.

In one embodiment, the invention relates to a de novo synthesized plasmid comprising at least a combination of a replication origin and a selection marker gene wherein

(a) the replication origin contains sequences relevant to autonomous plasmid replication in a host cell. In one preferred embodiment, the sequences relevant for autonomous plasmid replication are determined by prior art publications. In another preferred embodiment, the sequences relevant for autonomous plasmid replication are determined by experiments. In a preferred embodiment, the replication origin allows the plasmid to replicate in a low copy number in a host cell (less than 20 copies per cell). In another preferred embodiment, the replication origin allows the plasmid replication in a high copy number in a host cell (more than 200 copies per cell). In a further preferred embodiment, the replication origin allows the plasmid to replicate in an intermediate copy number in a host cell (from 20 to 200 copies per cell). In one preferred embodiment, the replication origin is from ColE1-type incompatibility group. In another preferred embodiment, it is from p15A-type incompatibility group. One skilled in the art will recognize that they can be from other types of origins as long as they allow autonomous plasmid replication in a host cell. For example, they can be from M13, pSC101, R6-5-type origins and the like; and

(b) the selection marker gene contains sequences relevant for allowing the selection of the plasmid in a host cell. In one preferred embodiment, the sequences relevant for allowing the selection of the plasmid are determined by prior art publications. In another preferred embodiment, the sequences relevant for allowing the selection of the plasmid are determined by experiments. The selection marker gene normally allows for phenotypic selection of the plasmid in a transformed host cells. The selection marker gene often encodes a product indicative of plasmid maintenance in a

host cell. Some selection marker genes are antibiotic resistance genes. In one preferred embodiment, the selection marker gene is ampicillin resistance gene. In another preferred embodiment, the selection marker gene is chloramphenicol resistance gene. In a further preferred embodiment, the selection marker gene is tetracycline resistance gene. In yet another preferred embodiment, the selection marker gene is kanamycin resistance gene. The selection marker gene contains a promoter, a ribosome binding site, a open reading frame encoding a protein product, a terminator and the like sequences necessary for allowing the selection of a host cell.

Wherein the de novo synthesized plasmid is not modified from the plasmid previously obtained from natural or recombinant sources.

The type of replication origin determines the compatibility of plasmids in a host cell. In applications such as protein co-expression, compatible plasmids from different replication origins of different compatibility groups are needed. The type of replication origin also determines the copy number of a plasmid. Within an incompatibility group, the copy number of a plasmid can be different. For example, pBR322 gives low copy number and pUC19 produces high copy number while they all belong to ColE1 incompatibility group. High copy number is advantageous for applications such as plasmid amplification and protein expression. However high copy number also depletes cellular resources and contributes cellular energy drain. Toxic DNA fragments are often difficult to be cloned and established in a high copy number plasmids. In some cases, proteins are expressed at a higher level in low copy number plasmids. Therefore low copy number plasmids are also needed. In one preferred embodiment, high copy number plasmids are selected. In another preferred embodiment, low copy number plasmids are selected. In a further preferred embodiment, the intermediate copy number plasmids are selected. The copy number of a plasmid can be estimated after the plasmid prep.

In one preferred embodiment, the de novo synthesized plasmid comprises of a replication origin with a selection marker gene. In another preferred embodiment, the de novo synthesized plasmid comprises of another replication origin with another selection

marker gene. In further preferred embodiment, the de novo synthesized plasmid comprises of a further different replication origin and a further different selection marker gene. These replication origins may be from same incompatibility group and with different copy numbers. They may also be from different incompatibility groups and with different copy numbers. These selection marker genes may be any genes that allow phenotypic selection of the plasmids in host cells. As will be understood by those of skill in the art that the replication origin and selection marker gene used to form the de novo synthesized plasmid can comprise of any combination.

In another preferred embodiment, the invention relates to a method of preparing a de novo synthesized plasmid combined from at least two DNA fragments comprising:

(a) preparing a linear replication origin DNA fragment;

(b) preparing a linear selection marker gene DNA fragment;

(c) combining the DNA fragments prepared in steps (a) and (b) to form a circular de novo synthesized plasmid;

(d) introducing the de novo synthesized plasmid made from step (c) into host cells; and

(e) selecting the plasmid with appropriate replication origin and selection marker from transformed host cells.

Wherein the linear replication origin DNA fragment contains sequences relevant to autonomous DNA replication. Wherein the linear selection marker gene DNA fragment contains sequences relevant to phenotypic selection of a plasmid in a host cell. Wherein any DNA fragment alone used for combining de novo synthesized plasmid cannot confer both autonomous DNA replication and selection to a plasmid. To minimize the cellular energy drain, the sequences with unknown and undesirable functions are minimized in

these DNA fragments. The DNA fragments containing the replication origin or selection marker gene may be prepared from restriction digested DNA or PCR reaction.

Wherein the replication origin and selection marker gene are combined together with or without linker DNA sequences. Linker DNA sequences are short DNA sequences necessary to link two or more DNA fragments together. In one preferred embodiment, the replication origin and selection marker gene are combined together by linker sequences designed for exonuclease-mediated cloning. In another preferred embodiment, the replication origin and selection marker gene are combined together by linker sequences designed for restriction enzyme-mediated cloning. In a further preferred embodiment, the replication origin and selection marker gene are combined together without any linker sequences by blunt end ligation. In yet another preferred embodiment, the replication origin and selection marker gene are combined together without any linker sequences by exonuclease-mediated cloning. In this case, homologous sequences are designed at the ends of the linear DNA fragments by synthetic oligos that the DNA fragments obtained by PCR with these oligos may anneal together after exonuclease treatment.

The linker DNA sequences are located between the replication origin and the selection marker gene. To minimize the cellular energy drain, the linker sequences should be as short as possible. The length of the linker sequences is the length necessary for efficient cloning. Depends on the particular method used to combine the DNA fragments, the length of linker sequences will vary. In one preferred embodiment, the length of linker sequences is 24 nucleotides. In another preferred embodiment, the length of linker sequences is 12 nucleotides. In yet another preferred embodiment, the length of linker sequences is at least the length of a restriction enzyme recognition site. In a further preferred embodiment, no linker is used for blunt end ligation. One skilled in the art would recognize that the linker length would be what it is needed to link the DNA fragments together.

It is convenient for the de novo synthesized plasmid to contain at least one restriction site. The restriction site will be used to linearize the plasmid, therefore it should be unique for

the plasmid. When no convenient restriction site is available, the plasmid may be linearized by PCR. When blunt end ligation is used, the DNA sequences can be designed that the ligated DNA may create a unique restriction site. In the case that the DNA fragments used to make these de novo synthesized plasmids contains restriction sites designed for plasmid linearization, these restriction sites may be mutated to make the linearization restriction site unique.

Wherein the combination of these DNA fragments can be achieved by annealing sequences produced by an exonuclease treatment. In one preferred embodiment, the sequences are produced by exonuclease III. In another preferred embodiment, the sequences are produced by lambda exonuclease. In a further preferred embodiment, the sequences are produced by an exonuclease activity of a DNA polymerase such as T4 DNA polymerase and the like. The annealing sequences can also be produced by restriction enzyme digestion. Wherein the combination of these DNA fragments can also be achieved by blunt end ligation.

The selection of the circular plasmids is achieved by adding an agent to the growth medium. The agent is usually an antibiotic. In one preferred embodiment, the antibiotic is ampicillin. In another preferred embodiment, the antibiotic is chloramphenicol. In a further preferred embodiment, the antibiotic is tetracycline. In yet another preferred embodiment, the antibiotic is kanamycin.

In a further preferred embodiment, the present invention also relates a method of using the de novo synthesized plasmid:

(a) linearizing the de novo synthesized plasmid;

(b) inserting one or more functional DNA fragments into the linearized plasmid to make other plasmids;

(c) introducing the plasmids made from step (b) into host cells;

(d) selecting the plasmids and host cells with desired properties; and

(e) using the plasmids and host cells for biomedical applications.

Wherein the de novo synthesized plasmid is linearized by restriction digestion. Wherein the de novo synthesized plasmid is linearized by PCR. Wherein the functional DNA fragments encode a promoter, a regulatory sequence, a ribosome binding site, restriction sites, a terminator, a polypeptide, a replication origin, a selection marker gene, and the like useful DNA sequences. To minimize the cellular energy drain, the sequences with unknown and undesirable functions are minimized in these DNA fragments. Wherein the desired properties are plasmid replication, selection, and other properties added by functional DNA fragments inserted from step (b). Wherein the biomedical applications are DNA cloning, DNA amplification, gene expression, gene therapy, DNA immunization, and the like.

A promoter is the DNA sequences where RNA polymerase binds. Promoters can be from prokaryote or eukaryote origin depends on the application of particular plasmid. Examples of the promoters include ara, lac, T7, tac, and trp from prokaryote, AOX1, GAL1, and TEF1 from yeast, MT, PH, and P10 from insect, CMV, RSV, and tk from eukaryotic cells.

A regulatory sequence allows regulated transcription of a gene. It often located immediately upstream or downstream of a promoter. A regulatory sequence often allows a transcription to be regulated by a highly selective chemical or by temperature changes. For example, the regulatory sequence for lac operator allows transcription by adding lactose or isopropy-beta-D-thiogalactopyronoside (IPTG).

A ribosome binding site is also known as the Shine-Dalgarno (SD) sequence. It binds to ribosome allowing translation initiation. Following ribosome binding site is the translation initiation codon ATG. Multiple restriction sites, which are unique to the

plasmid, permit insertion of an exogenous gene and verification of plasmid construction. An open reading frame of a polypeptide is placed between the translation initiation codon ATG and translation termination codons TAA, TAG or TGA. Sometimes multiple termination codons are placed at all reading frames to ensure effective translation termination from all reading frames.

A terminator is required for transcription termination and poly A addition for eukaryote. It also provides stability of the RNA transcripts. A terminator often located at 3' end of a gene. However a terminator can be placed anywhere on a plasmids to prevent transcription leakage from upstream promoters. Examples of terminators include tHP, T7, and rrnB from prokaryote, TK, BGH, and SV40 poly A addition sites from eukaryote.

Plasmids may be introduced into host cells by various methods known in recombinant techniques. In one preferred embodiment, calcium chloride method is used. In another preferred embodiment, rubidium chloride method is used. In a further preferred embodiment, electroporation method is used. As those skilled in the art will recognize, host cells containing these plasmids can be produced by a number of means such as transformation, transduction, transfection, conjugation, and the like.

Plasmid amplification and protein expression may be performed using standard recombinant DNA techniques as taught herein or as known in the art. Many of the techniques are described in J. Sambrook et al., Molecular cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., 1989, Coligan et al., Current Protocols in Protein Science, Current Protocols, U. S. A., 1995, and Ausubel et al., Current protocols in Molecular Biology, Current protocols, U. S. A. 1994.

Host cells containing various plasmids may be used to aid other protein expression as known in the art. In one preferred embodiment, host cells containing plasmids aid the yield of a target protein expression. In another preferred embodiment, host cells containing plasmids aid the solubility of a target protein expression. In a further preferred

embodiment, host cells containing plasmids modify the expressed target protein. In yet another preferred embodiment, host cells containing plasmids stabilize the expressed target protein.

It should be understood that not all functional DNA fragments would function equally well for a particular application. Neither will all host cells function equally well with the same plasmid. However, one of ordinary skill in the art may make a selection among the DNA fragments and host cells using the guidance provided herein without undue experimentation and without departing from the scope of the present invention.

The invention is described in further detail in the following non-limiting examples.

EXAMPLE 1

Construction of a de novo synthesized plasmid

Prepare a linear DNA fragment containing replication origin.
Oligos 1 and 4 (sequence ID NOS: 1 and 4) were synthesized for replication origin based on pACYC 177 (Chang et al., J. Bacteriol., 134, 1141-1156 (1978)). Polymerase chain reaction (PCR, Mullis et al., and U. S. PAT. No. 4,683,202) was used to generate DNA fragments containing the replication origin. One nanogram of pACYC177 was used as templates. One microgram each of oligos were used as primers in each reaction. Total volume of each reaction was 100 microliters. Five units of Taq DNA polymerase were used in each reaction. The following temperature was indicated in Celsius. PCR were performed at cycling condition of 94 degree for 30 seconds, 68 degree for 2 minutes, and 72 degree for 2 minutes. These cycling conditions were repeated for 40 times. The reactions were kept at 72 degree for 5 minutes. Then the reactions were hold at 4 degree. The PCR product was verified by agarose gel electrophoresis. The replication origin on this DNA fragment was arbitrarily named origin 4 (4).

Prepare a linear DNA fragment containing selection marker gene.

Oligos 7 and 11 (sequence ID NOS 7 and 11) were synthesized for selection marker gene conferring tetracycline resistance. PCR was used to prepare the tetracycline selection marker gene using pBR 322 as a template (Bolivar et al., Gene 2, 75-93 (1977)). The PCR conditions were same as described above. The selection marker gene on this DNA fragment was named tetracycline (T).

The DNA fragments containing replication origin and selection marker gene were prepared. None of these DNA fragments alone can confer both autonomous DNA replication and host cell selection. Linker sequences were designed on each of these oligos to facilitate cloning. Only G and C bases are designed in the linker to give stable annealing. A Sma I site is designed on one of these linker sequences. The Sma I site can be used to linearize the plasmid in the future.

DNA fragments containing above described replication origin and selection marker gene were combined together by exonuclease III mediated cloning (Li et al., Nucleic Acid Res. 25:4165-4166 (1997)) to form a de novo synthesized plasmid. The combined DNA fragments were transformed into DH5a cells. The transformed cells were selected on Luria-Berani (LB) agar plate containing 12.5 microgram per milliliter tetracycline. The size of the plasmid was verified by restriction digestion and agarose gel electrophoresis. The de novo synthesized plasmid was named p4T since it is made from replication origin 4 (4) and tetracycline (T) selection marker. Schematic diagrams of construction of the de novo synthesized p4T are shown on Fig. 1.

EXAMPLE 2

Construction of other de novo synthesized plasmids

Sixty de novo synthesized plasmids were constructed as described above. Ten of them were used in the future plasmid construction. They are p1A, p3A, p1C, p2C, p3C, p4C, p2T, p4T, p2K, and p4K (sequence ID NOS: 32 to 41). The Arabic number of the plasmid represents its replication origin and the capital letter of the plasmid represents its

selection marker gene. For example the plasmid p4C contains replication origin 4 and selection marker gene resistant to chloramphenicol. These plasmids are the smallest known plasmids containing same replication origins and selection marker genes. Since the odd numbered plasmids contain replication origins based on ColE1 and the even numbered plasmids contain replication origins based on p15A, the odd numbered plasmids should be compatible with the even numbered plasmids. Plasmids based pBR322 and p15A replication origins should be low copy number plasmids and plasmids based on pUC19 replication origin should be high copy number plasmids.

It is surprising to find that p4T is a high copy number plasmid since replication origin 4 is based on p15A replication origin which is considered to be a low copy number origin (Sambrook et al., Molecular cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., Vol. 1, pp1.3-1.5, (1989)). Sixteen de novo synthesized plasmids were prepared under same conditions. They are linearized by Sma I restriction digestion and run on 0.8% agarose gel. The picture of this gel is shown on Fig 2. The names of these plasmids are listed above the gel. The marker is 1 Kb DNA Ladder (Life Technologies, Rockville, MD). The arrow on the gel indicates approximate position of the linearized plasmid. Consistence with prior art teaching, p2A, p2T, and p2K are low copy number plasmids since their replication origins are based on p15A origin. Surprisingly, the copy number of p4T is comparable with plasmids p3A, p3T, and p3K based on high copy number plasmid pUC19.

Restriction sites such as BamH I, EcoR I, EcoR V, Hind III, Cla I, Pst I, Nco I, Nde I, Nsi I, Pvu II, Sca I, Sal I, Sph I, Sma I, and Xho I on these plasmids were mutated by site directed mutagenesis (Current protocols in Molecular Biology, Ausubel et al., Current protocols, U. S. A., (1994)).

Oligos 1 to 4 (sequence ID NOS: 1 to 4) were synthesized for replication origins of these ten plasmids. Replication origins of CoE1 based pBR322 (Bolivar et al., Gene 2, 75-93 (1977)) and pUC19 (Vieira et al., Gene 19, 259-268 (1982)) and p15A based pACYC177 (Chang et al., J. Bacteriol., 134, 1141-1156 (1978)) were used in the experiments. The

sequences required for these replication origins are described in the previous papers (Bird et al., J. Bacteriol., 145, 1305-1308 (1981), Stuber et al., Proc. Natl. Acad. Sci. USA, 78, 167-171 (1981), Marians et al., J. Biol. Chem., 257, 5656-5662 (1982), Selzer et al., Cell 32, 119-129 (1983)). PCR with oligo 2, oligo 4, and pBR322 produced replication origin 1 DNA fragment based on ColE1. PCR with oligo 3, oligo 4, and pACYC177 produced replication origin 2 DNA fragment based on p15A. PCR with oligo 1, oligo 4, and pUC19 produced replication origin 3 DNA fragment based on ColE1. PCR with oligo 1, oligo 4, and pACYC177 produced replication origin 4 DNA fragment based on p15A.

Oligos 5 to 12 (sequence ID NOS: 5 to 12) were synthesized for selection marker genes of these ten plasmids. Antibiotics resisted by host cells containing these selection marker genes are ampicillin, tetracycline, chloramphenicol, and kanamycin. The promoters and terminators of these selection marker genes were studied in the previous papers (Stuber et al., Proc. Natl. Acad. Sci. USA, 78, 167-171 (1981), Brosius et al., J. Biol. Chem., 257, 9205-9210 (1982), Sheflin et al., Nucleic Acid Res., 13, 6137-6154 (1985), Smith et al., Gene 84, 159-164 (1989)). Oligos 5 to 12 were designed according to these studies. PCR with oligo 5, oligo 9, and pUC19 produced ampicillin DNA fragment that will confer ampicillin resistance. PCR with oligo 8, oligo 12, and pACYC184 produced chloramphenicol DNA fragment that will confer chloramphenicol resistance. PCR with oligo 7, oligo 11, and pBR322 produced tetracycline DNA fragment that will confer tetracycline resistance. PCR with oligo 6, oligo 10, and pACYC177 produced kanamycin DNA fragment that will confer kanamycin resistance.

EXAMPLE 3

Construction of other plasmids generated from de novo synthesized plasmids

Hundreds of other plasmids are generated from the ten de novo synthesized plasmids described above. Construction of p4TI3E and p2CIXL will be described as examples below. Plasmid p4TI3E contains replication origin 4 (4), a selection marker gene resistant to tetracycline (T), a LacI gene repressing a promoter containing LacI operator (I), an

artificial transcription unit 3 (3), and DNA sequences encoding GroEL and GroES proteins (E). Plasmid p2CIXL contains replication origin 2 (2), a selection marker gene resistant to chloramphenicol(C), a LacI gene repressing a promoter containing Lac I operator (I), and human retinoid X receptor beta ligand binding domain (XL, Marks et al., EMBO J. 11, 1419-1435 (1992)).

Oligos 13 and 14 (sequence ID NOS: 13 and 14) were synthesized for LacI gene (Farabaugh, Nature 274, 765-769 (1978)). One nanogram of pGEX-3X (Smith et al., Gene 67, 31-39 (1988)) was used as template. The PCR condition was same as that used for replication origins and selection marker genes. The PCR product was cloned into Sma I site of p4T to make plasmid p4TI.

Oligos 15 to 24 (sequence ID NOS: 15 to 24) were synthesized for the artificial transcription unit 3. Oligos 15 to 24 were mixed at concentration of 10 nanogram per microliter. 10 microliters of this oligo mix was used for first PCR with 5 microliter water, 2 microliter PCR buffer, 3 microliter 1 mM dNTPs, and 1 unit Taq DNA polymerase. The PCR was performed at cycling conditions of 94 degree for 30 seconds, 68 degree for 30 seconds, and 72 degree for 30 seconds. These cycling conditions were repeated for 40 times. The reaction was kept at 72 degree for 5 minutes. Then the reaction was hold at 4 degree. Five microliters of this PCR product was used as template for second PCR. Second PCR was performed with 1 microgram each of oligo 15 and 24, 5 microliters of 1 mM dNTPs, and 5 units of Taq in total volume of 50 microliters. The cycling conditions were same as the first PCR. The artificial transcription unit 3 (sequence ID NO: 25) was obtained. Terminator tHP (Nohno et al., J. Bacteriol. 170, 4097-4102 (1988)) was placed at the 5' end of the transcription unit to prevent transcription leakage from upstream promoters. The promoter, terminator, and ribosome binding site of bacterial phage T7 gene 10 (Dunn et al., J. Mol. Biol., 166, 477-535 (1983)) were used in the transcription unit for high level protein expression. LacI operator sequences (Calos, Nature 274, 762-765 (1978)) were placed immediately after the T7 promoter. With another LacI operator located at 3' end of LacI gene, these operators will assure minimum basal level expression and maximum induction upon adding IPTG. Multiple

cloning sites were placed after the ribosome binding site. These multiple cloning sites contain Nde I, Hind III, Sma I, Kpn I, Sal I, Spe I, and Hpa I restriction sites. Multiple stop codons are present in all reading frames after the multiple cloning sites. Two linkers of 12 nucleotides each were placed at 5' and 3' ends of the artificial transcription unit 3. The transcription unit 3 was cloned into Sma I site of plasmid p4TI to generate p4TI3.

Oligos 26 to 29 (sequence ID NOS: 26 to 29) were synthesized for GroE gene (Hemmingsen et al., Nature 333, 330-334 (1988)). Two proteins GroES and GroEL are encoded by GroE gene. One microgram each of oligos 26 and 27 with 0.1 microgram of genomic DNA of E.coli strain W3110 were used for first PCR. The PCR was performed with 1 unit each of Taq and Vent DNA polymerase (New England Biolabs, Beverly, Maryland) in total volume of 20 microliters. Cycling conditions were 94 degree for 30 seconds, 60 degree for 30 seconds, and 72 degree for 2 minutes. These cycling conditions were repeated for 60 times. The reaction was kept at 72 degree for 5 minutes. Then the reaction was hold at 4 degree. One microliter of the PCR product was used for second PCR. Second PCR was performed with 1 microgram each of oligo 28 and 29, 10 microliters of 1 mM dNTPs, and 1unit each of Taq and Vent in total volume of 50 microliters. The cycling conditions were same as the first PCR. This PCR product was cloned into the Nde I and Sal I sites of plasmid p4TI3 to generate p4TI3E. The schematic diagrams of these plasmid construction are shown on Fig. 3.

Oligos 30 and 31 (sequence ID NOS: 30 and 31) were synthesized to make p2CXL. Ten nanograms of pET-15b-RXR LBD (Li et al., Proc. Natl. Acad. Sci. USA 94, 2278-2283 (1997)) was used as template in the PCR. One microliter each of 50 uM oligos 30 and 31, 1 unit each of Taq and Pfu (Stratagene, La Jolla, California), and 6 microliters of 1 mM dNTPs were used in the total volume of 20 microliters. Cycling conditions were 94 degree for 45 seconds, 68 degree for 30 seconds, and 72 degree for 2 minutes. These cycling conditions are repeated for 35 times. Then the reaction was hold at 4 degree. The PCR product was cloned into Sma I site of p2C to make p2CXL.

EXAMPLE 4

Protein expression of plasmids generated from de novo synthesized plasmids

Plasmids p4TI3E and p2CXL were introduced to E.coli strain BL21 (DE3) by calcium chloride method. Colonies containing these plasmids were obtained from LB agar plates containing ampicillin or chloramphenicol respectively. One colony from each plate was inoculated into LB media. Incubate them at 37 degree with shaking for 3 to 5 hours until their OD600 reaches 0.4 to 1.0. Take out 100 microliters each as non-induced sample. Add IPTG to final concentration of about 1 mM/ml. Incubate them at 37 degree with shaking for 1 hour 40 minutes and 2 hour 30 minutes respectively. Take out 100 microliters each as induced sample. Spin down the non-induced and induced samples at 14,000 RPM for 1 minute. Resuspend the cell pellets in 20 ul 1X SDS buffer. Boil them for 3 minutes. Load 5 microliters each on 12% Tris-glycine SDS gel. After electrophoresis, the gel was stained for 10 minute with Coomassie blue staining solution (0.25% Coomassie brilliant blue R-250, 10% acidic acid, 45% methanol). Detain the gel with 7.5% methanol and 5% acidic acid overnight (about 16 hours). Both proteins GroEL (EL) and GroES (ES) were expressed from induced sample of E.coli cells containing p4TI3E (Fig. 4A). Protein RXR LBD (XL) was expressed from induced sample of E.coli strain containing p2CXL (Fig. 4B).

EXAMPLE 5

Host cells containing plasmids generated from the de novo synthesized plasmid aid the solubility of another protein that is not soluble when expressed by itself

A plate of host cells containing p2CXL plasmids were obtained from EXAMPLE 3. Host cells containing plasmid pET-15b-RAR-LBD (Li et al., Proc. Natl. Acad. Sci. USA 94, 2278-2283 (1997)) were plated on a Laurie Broth agar plate with ampicillin. Plasmid pET-15b-RAR-LBD was introduced into the host cells containing p2CXL. Plasmids p2CXL and pET-15b-RAR-LBD contain compatible replication origins and different selection markers (ampicillin and chloramphenicol). Therefore they can be selected and

co-exist in one cell. The cells were grown on plate containing both ampicillin and chloramphenicol. Host cells containing p2CXL and pET-15b-RAR-LBD were grown on plates containing chloramphenicol and ampicillin respectively. One colony from each plate was picked up and inoculated into LB media with appropriate antibiotic(s). The concentrations of ampicillin and chloramphenicol are 50 mM per milliliter (mM/ml) and 35 mM/ml respectively. These cultures were incubated at 37 degree with shaking until their OD600 reaches 0.4 to 1.0. The cultures were induced by IPTG at final concentration of 1 mM/ml for 2 hours and 30 minutes. The cells were harvested and sonicated in HKI buffer (20 mM Hepes, pH 8.0, 100 mM KCL, 20 mM imidazole). Soluble and insoluble proteins were obtained from supernatants and pellets respectively. Binding to nickel agarose beads and by washing multiple times with HKI buffer purifies these proteins. The soluble, insoluble, purified proteins were loaded on Tris-glycine SDS gels. After electrophoresis, the gels were stained, destained, and dried between cellophane membranes. It has been shown that RXR LBD (XL) is soluble and RAR LBD (AL) is largely insoluble and can hardly purified when they expressed individually (Li et al., Proc. Natl. Acad. Sci. USA 94, 2278-2283 (1997)). Significant amount of AL becomes soluble and purified when it is expressed in the host cells containing p2CXL plasmids (Fig.5). Therefore host cells containing plasmids p2CXL generated from the de novo synthesized p2C aid the solubility of AL.

All publications mentioned hereinabove are hereby incorporated their entirety by reference.

While the foregoing invention has been described in some detail for purpose of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.